

Technical Note

PAXgene® Blood ccfDNA Tube (CE-IVD) Sample Transportation Study (Summer Profile)

The objective of this study was to test the effect of transport on whole blood samples collected into PAXgene Blood ccfDNA Tubes (CE-IVD). In the study, the preanalytical steps including handling, storage, processing and documentation were conducted according to ISO 20186-2:2019 and ISO 20186-3:2019 (Molecular in vitro diagnostic examinations – Specifications for pre-examination processes for venous whole blood – Part 2: Isolated genomic DNA and Part 3: Isolated circulating cell-free DNA from plasma).

Introduction

The PAXgene Blood ccfDNA Tube (PAXgene Tube) is a sterile, single use, plastic, evacuated blood collection tube with a ccfDNA stabilization additive (1.5 ml of liquid additive) and has a nominal blood draw volume of 10 ml. The tube additive is non-crosslinking, free of formaldehyde-releasing substances, stabilizes blood cells, and prevents apoptosis. Blood-filled PAXgene Blood ccfDNA Tubes can be stored for up to 10 days at 2–25°C, up to 7 days at 2–30°C, or up to 3 days at 2–37°C prior to processing. Plasma is separated from the nucleated cellular fraction by a double centrifugation, first for 15 minutes at 1,600–3,000 × *g*, then transfer of the plasma into a secondary tube, followed by centrifugation of the plasma for another 10 minutes at 1,600–3,000 × *g*.

Plasma generated from whole blood collected into PAXgene Blood ccfDNA Tubes (CE-IVD) can be used to process ccfDNA automated with the PreAnalytiX® QIASymphony® PAXgene Blood ccfDNA Kit (CE-IVD), or manually with the QIAGEN® QIAamp® DSP Circulating Nucleic Acid Kit (CE-IVD). The nucleated cellular fraction or buffy coat remaining after removal of the plasma can be processed for genomic DNA (gDNA) automated with the QIAGEN QIASymphony DSP DNA Mini and Midi Kits (CE-IVD) or manually with the QIAGEN QIAamp DSP DNA Blood Mini Kit (CE-IVD).

PreAnalytiX developed the PAXgene Blood ccfDNA Tube (CE-IVD) to minimize post-collection changes of circulating, cell-free DNA (ccfDNA) and genomic DNA profiles in whole blood caused by preanalytical workflow variables (**Figure 1**).

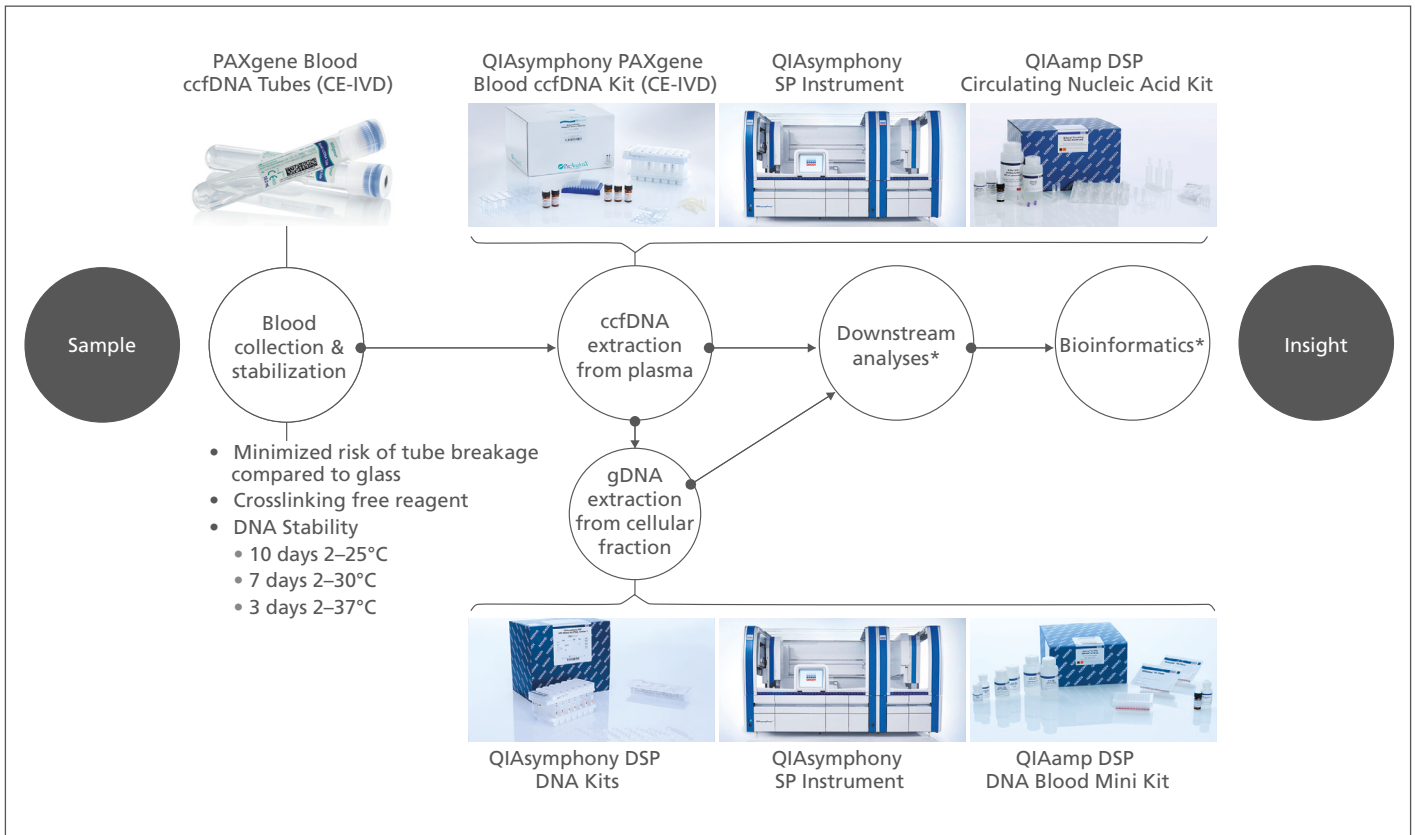


Figure 1. PAXgene Blood ccfDNA workflow (CE-IVD).

The PAXgene Blood ccfDNA Tube (CE-IVD) with the QIASymphony PAXgene Blood ccfDNA Kit (CE-IVD) and QIAGEN QIASymphony instrument (CE-IVD) have been verified and validated as an integrated workflow.

Plasma generated from whole blood collected into PAXgene Blood ccfDNA Tubes (CE-IVD) can be used to process ccfDNA automated with the QIASymphony PAXgene Blood ccfDNA Kit, or manually with the QIAamp DSP Circulating Nucleic Acid Kit. The nucleated cellular fraction or buffy coat remaining after removal of the plasma can be processed for gDNA automated with the QIASymphony DSP DNA Mini and Midi Kits, or manually with the QIAamp DSP DNA Blood Mini Kit.

*Only when used in combination with products for In Vitro Diagnostic use.

The PAXgene Blood ccfDNA workflow is intended to standardize the preanalytical workflow from blood collection, stabilization, and transport until purification of ccfDNA from plasma and gDNA from the nucleated cellular fraction.

The PAXgene Blood ccfDNA Tube (CE-IVD) is CE marked for In Vitro Diagnostic (IVD) use according to the EU Regulation on in vitro diagnostic medical devices (REGULATION (EU) 2017/746) and as part of IVD development has been intensively tested in various verification and validation studies.

In this technical note, we present the results from a full workflow verification study including all the preanalytical steps from sample collection until the purified analyte is ready for analysis. This included transportation of whole blood by air and ground shipment methods, as well as different sites for collection, plasma processing, and DNA purification.

Study Design

Blood samples from 29 consented, apparently healthy adult subjects were collected into two lots of PAXgene Blood ccfDNA Tubes (CE-IVD) at QIAGEN (Hilden, Germany). Samples from one additional donor were withdrawn from the study due to insufficient fill volume. One tube per lot from each donor was processed immediately (≤ 6 hours) at day 0 (T_0) or stored for 7 days at 30°C, the longest storage duration specified for an elevated temperature above room temperature. One additional tube per lot was transported from QIAGEN to BD (Franklin Lakes, USA) via domestic ground and international air transportation. For the transport, the blood samples were packaged to be IATA-compliant, following DGR (Dangerous Goods Regulation) Packing Instruction 650 for transport of Biological Substances Category B (UN3373). This included a leakproof triple packaging concept with a primary receptacle, a secondary package, and a rigid outer package. No cooling devices were included in the package. To measure temperature fluctuations during transport, a data logger was included.

Upon arrival, samples were processed by a double centrifugation at room temperature with brake set to medium: 15 min $1,900 \times g$, transfer of the plasma into a secondary tube, followed by 10 min $1,900 \times g$, according to the instructions for use for the PAXgene Blood ccfDNA Tube (CE-IVD), to separate the plasma from the nucleated cellular fraction. Both plasma and residual nucleated cellular fraction were frozen at -20°C and sent back to QIAGEN on dry ice (Figure 2). Before freezing, to assess the level of hemolysis, a small aliquot of plasma sample was removed from each sample to measure plasma-free hemoglobin (PFHb) on the Beckman Coulter UniCel DxC 680i instrument, according to the instructions of the manufacturer.

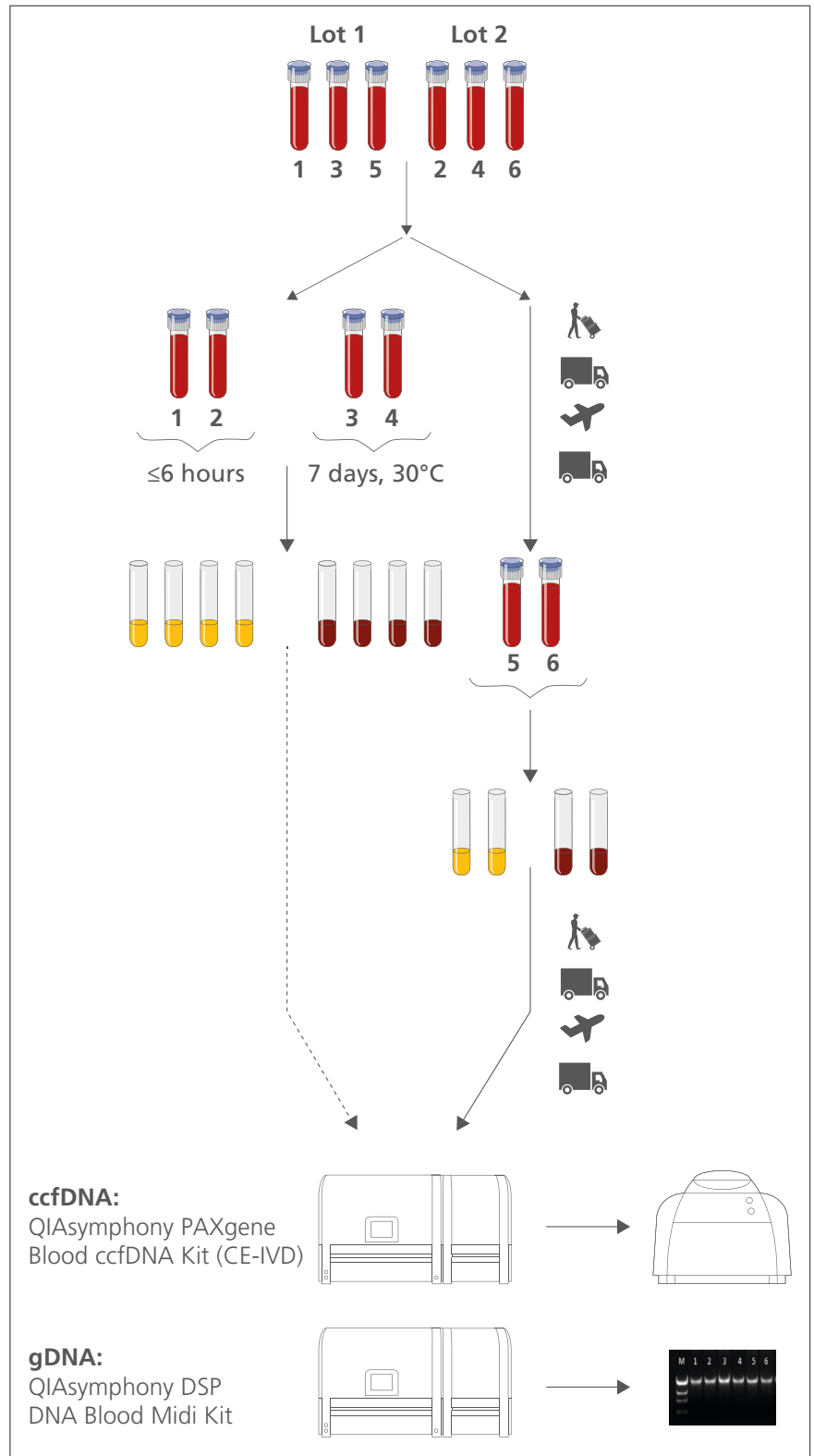


Figure 2. Workflow for the Transport Study.

ccfDNA was extracted from the plasma received at QIAGEN with the QIASymphony PAXgene Blood ccfDNA Kit (CE-IVD) on the QIASymphony SP instrument using the 2.4 ml protocol. Relative ccfDNA yield was quantified by a probe-based real-time PCR assay amplifying a 66 bp fragment of the 18S rDNA gene on the QIAGEN Rotor-Gene® Q MDx instrument.

gDNA was extracted from the nucleated cellular fraction received at QIAGEN with the QIASymphony DSP DNA Midi Kit (CE-IVD) on the QIASymphony SP instrument using the 400 µl protocol. gDNA yield and purity were measured by spectrophotometry on a NanoDrop™ spectrophotometer (Thermo Fisher Scientific), and gDNA integrity by agarose gel electrophoresis.

Results

The temperature within the package during transport of blood samples was tracked with a data logger (Testo, Germany). It varied between 18 to 35°C, with the highest value occurring during ground transportation in the US from Newark International Airport to the final destination at BD (Franklin Lakes, USA) (**Figure 3**). The temperature range and time between blood collection and processing were within the specifications for the PAXgene Blood ccfDNA Tube (CE-IVD).

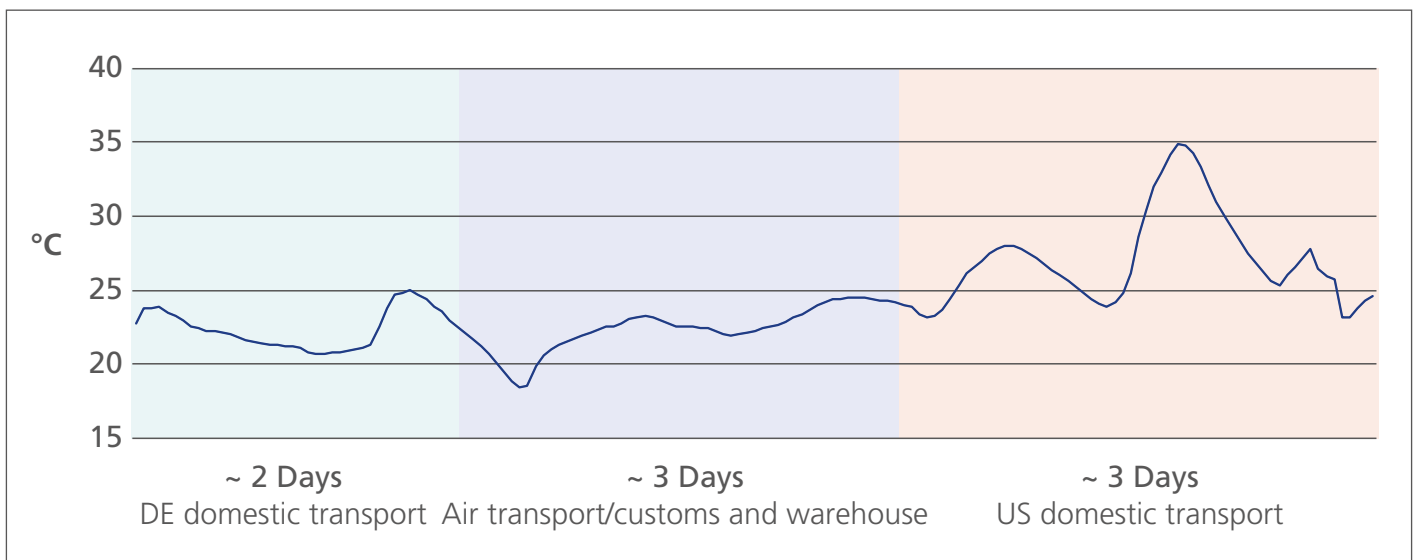


Figure 3. Temperature during Summer Transport of whole blood samples collected into PAXgene Blood ccfDNA Tubes (CE-IVD) from QIAGEN (Hilden, Germany) to BD (Franklin Lakes, USA).

The level of plasma-free hemoglobin in PAXgene Blood ccfDNA Tube (CE-IVD) plasma samples after transportation was higher for some samples compared to the plasma from samples processed directly after blood draw, but comparable to samples stored for 7 days at 30°C, with a median of ~40 mg/dl (**Figure 4 A**). In contrast, as shown previously, unstabilized blood samples from healthy donors collected into K₂EDTA Tubes show increased hemolysis when stored for 7 days at 30°C (**Figure 4 B**).

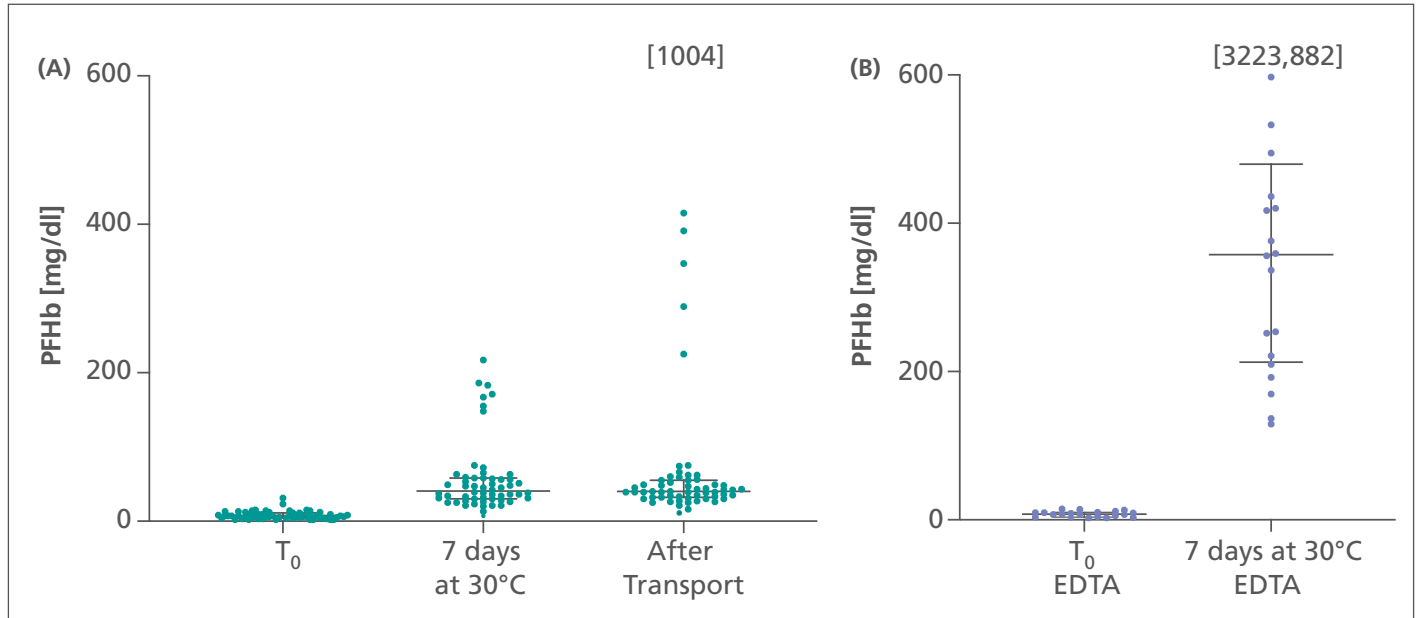


Figure 4. Plasma-free hemoglobin (PFHb) measured on the Beckman Coulter instrument (UniCel DxC 680i).

(A) Concentration of PFHb in blood samples from 29 healthy donors collected into two lots of PAXgene Blood ccfDNA Tubes (CE-IVD), processed immediately (T₀), stored for 7 days at 30°C, or transported from QIAGEN (Hilden, Germany) to BD (Franklin Lakes, USA). (B) Concentration of PFHb in blood samples from 20 healthy donors collected into K₂EDTA spray dried tubes processed immediately (T₀), or stored for 7 days at 30°C. Medians and the 25th and 75th percentiles are denoted.

ccfDNA was purified on the fully automated QIASymphony SP instrument with the QIASymphony PAXgene Blood ccfDNA Kit (CE-IVD). Yield, measured as copies of 18S rDNA molecules per ml plasma, was slightly increased in plasma from transported samples compared to T₀ samples, but wasn't significantly different from the corresponding samples stored for 7 days at 30°C (**Figure 5**).

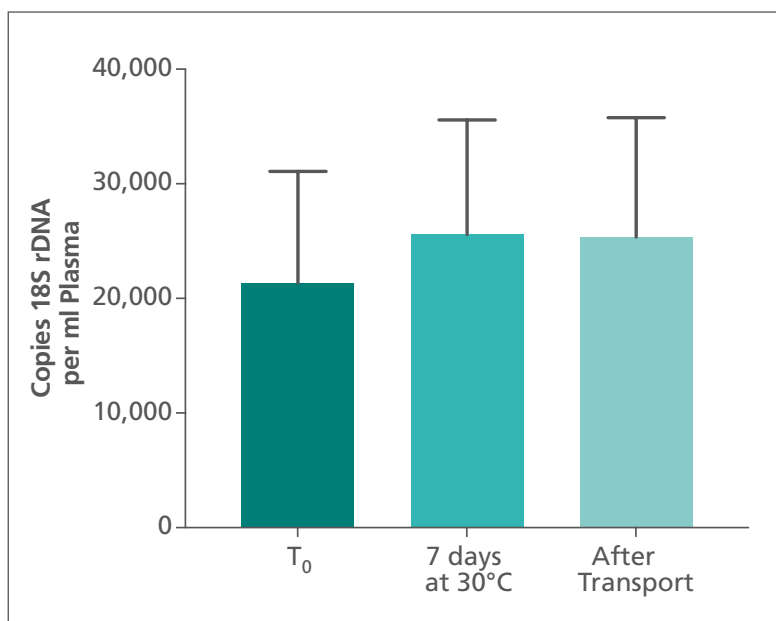


Figure 5. Copies of 18S rDNA per ml plasma in blood samples from 29 healthy donors collected into two lots of PAXgene Blood ccfDNA Tubes (CE-IVD), processed immediately (T₀), stored for 7 days at 30°C or transported from QIAGEN (Hilden, Germany) to BD (Franklin Lakes, USA).

ccfDNA was extracted with the QIASymphony PAXgene Blood ccfDNA Kit (CE-IVD) on the QIASymphony SP instrument using the 2.4 ml protocol. Quantification with probe-based validated qPCR assay (target 18S rDNA gene, 66 bp amplicon) on the Rotor-Gene Q instrument. Values are means with standard deviation.

In general, genomic DNA isolated from the nucleated cellular fraction was of high yield, purity, and integrity. A slight drop in yield could be observed between samples stored for 7 days at 30°C or transported compared to samples from day 0 (T_0). With regard to purity and integrity, no significant difference could be observed between all conditions tested (**Figure 6**).

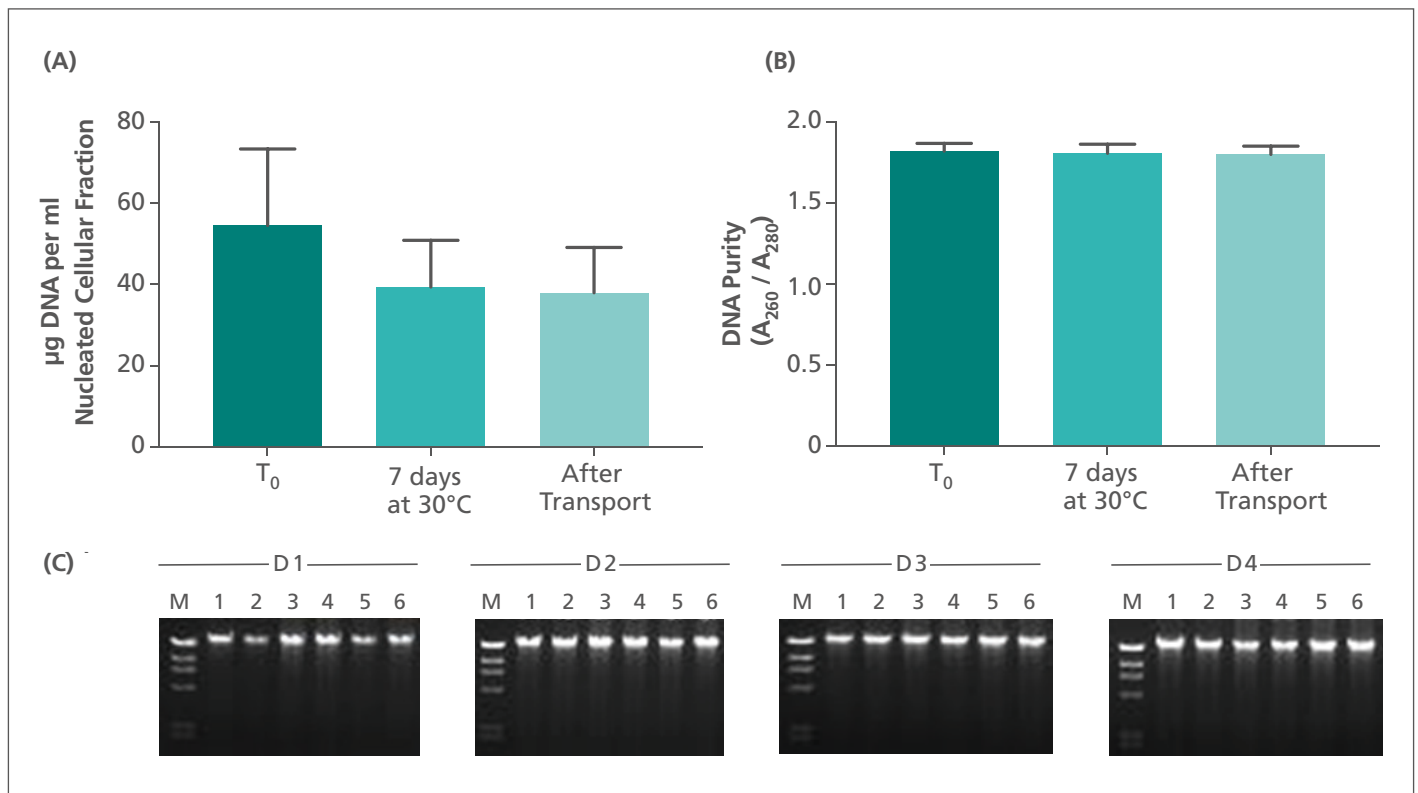


Figure 6. Yield, purity and integrity of genomic DNA from the nucleated cellular fraction of 29 healthy donors collected into two lots of PAXgene Blood ccfDNA Tubes (CE-IVD), processed immediately (T_0), stored for 7 days at 30°C, or transported from QIAGEN (Hilden, Germany) to BD (Franklin Lakes, USA).

DNA was extracted with the QIASymphony DSP DNA Midi Kit (CE-IVD) on the QIASymphony SP instrument using the 400 µl protocol. **(A)** gDNA yield in µg DNA per ml nucleated cellular fraction and **(B)** gDNA purity (A_{260} / A_{280}); both yield and purity were measured by spectrophotometry on a NanoDrop spectrophotometer. Values are means with standard deviation. **(C)** DNA integrity using agarose gel electrophoresis is shown for four Donors (D1-D4). Lane 1,2 DNA from T_0 ; lane 3,4 DNA from 7 days 30°C; lane 5,6 DNA after transport; a Lambda x Hind III marker was loaded into lane "M". The upper band of this marker represents a DNA fragment of 23 kb.

Conclusion

The PAXgene Blood ccfDNA workflow consists of CE-IVD blood collection tube and DNA preparation kits and covers the whole preanalytical workflow from blood collection to DNA purification. Blood samples in the PAXgene Blood ccfDNA Tube (CE-IVD) are stabilized and can be stored or transported within the specified duration and temperature range if immediate centrifugation of the blood collection tube is not possible. Red blood cell breakdown is minimized, which results in reduced hemolysis. Genomic DNA degradation and release from white blood cells is minimized which leads to unchanged concentrations of ccfDNA in plasma as well as consistent high-quality genomic DNA from the nucleated cellular fraction.

For optimal results it is highly recommended to follow the instructions for use for the PAXgene Blood ccfDNA Tube (CE-IVD).

Products used

Product	Catalog No.
PAXgene Blood ccfDNA Tube (CE-IVD) (100)	768165
QIASymphony PAXgene Blood ccfDNA Kit (CE-IVD) (192)	768566
QIASymphony DSP DNA Midi Kit (96) (QIAGEN)	937255
QIASymphony SP instrument (QIAGEN)	9001297
QIAamp DSP Circulating Nucleic Acid Kit (50) (QIAGEN)	61504
Rotor-Gene Q instrument (QIAGEN)	9001550



For up-to-date licensing information and product-specific disclaimers, see the PreAnalytiX website www.preanalytix.com/trademarks-disclaimers) and respective PreAnalytiX product webpages. PreAnalytiX instructions for use and handbooks are available at www.preanalytix.com, eifu.bd.com, www.qiagen.com, or can be requested from BD or QIAGEN Technical Services or your local distributor.

The PAXgene Blood ccfDNA Tube (IVD) and QIASymphony PAXgene Blood ccfDNA Kit (IVD) are distributed by BD and QIAGEN and their distributors and are not available in certain countries including the US. Please visit www.preanalytix.com or contact your local supplier for more details and product availability.

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 NanoDrop™ (Thermo Fisher Scientific)

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Technical Note

PAXgene[®] Blood ccfDNA Tube (CE-IVD) Plasma and Nucleated Cellular Fraction Stability Study

The objective of this study was to test plasma and nucleated cellular fraction stability generated from blood collected into PAXgene Blood ccfDNA Tubes (CE-IVD). In the study, the preanalytical steps including handling, storage, processing and documentation were conducted according to ISO 20186-2:2019 and ISO 20186-3:2019 (Molecular in vitro diagnostic examinations – Specifications for pre-examination processes for venous whole blood – Part 2: Isolated genomic DNA and Part 3: Isolated circulating cell-free DNA from plasma).

Introduction

The PAXgene Blood ccfDNA Tube (PAXgene Tube) is a sterile, single use, plastic, evacuated blood collection tube with a circulating, cell-free DNA (ccfDNA) stabilization additive (1.5 ml of liquid additive) and a nominal blood draw volume of 10 ml. The tube additive is non-crosslinking, free of formaldehyde-releasing substances, stabilizes blood cells and prevents apoptosis. Blood filled in PAXgene Blood ccfDNA Tubes can be stored for up to 10 days at 2–25°C, up to 7 days at 2–30°C, or up to 3 days at 2–37°C prior to processing. Plasma is separated from the nucleated cellular fraction by a double centrifugation, first for 15 minutes at 1,600–3,000 × *g*, then transfer of the plasma into a secondary tube, followed by centrifugation of the plasma for another 10 minutes at 1,600–3,000 × *g*. As an alternative, after a first centrifugation for 15 minutes at 3,000 × *g*, the tube can be directly placed on the QIAGEN[®] QIASymphony[®] SP instrument for automated ccfDNA extraction.

Plasma generated from whole blood collected into PAXgene Blood ccfDNA Tubes (CE-IVD) can be used to process ccfDNA automated with the PreAnalytiX[®] QIASymphony PAXgene Blood ccfDNA Kit (CE-IVD), or manually with the QIAGEN QIAamp[®] DSP Circulating Nucleic Acid Kit. The nucleated cellular fraction or buffy coat remaining after removal of the plasma can be processed for genomic DNA (gDNA) automated with the QIAGEN QIASymphony DSP DNA Mini and Midi Kits or manually with the QIAGEN QIAamp DSP DNA Blood Mini Kit.

PreAnalytiX developed the PAXgene Blood ccfDNA workflow to standardize the preanalytical workflow in order to minimize post-collection changes in circulating, cell-free DNA (ccfDNA) and gDNA profiles of whole blood caused by preanalytical variables (**Figure 1**).

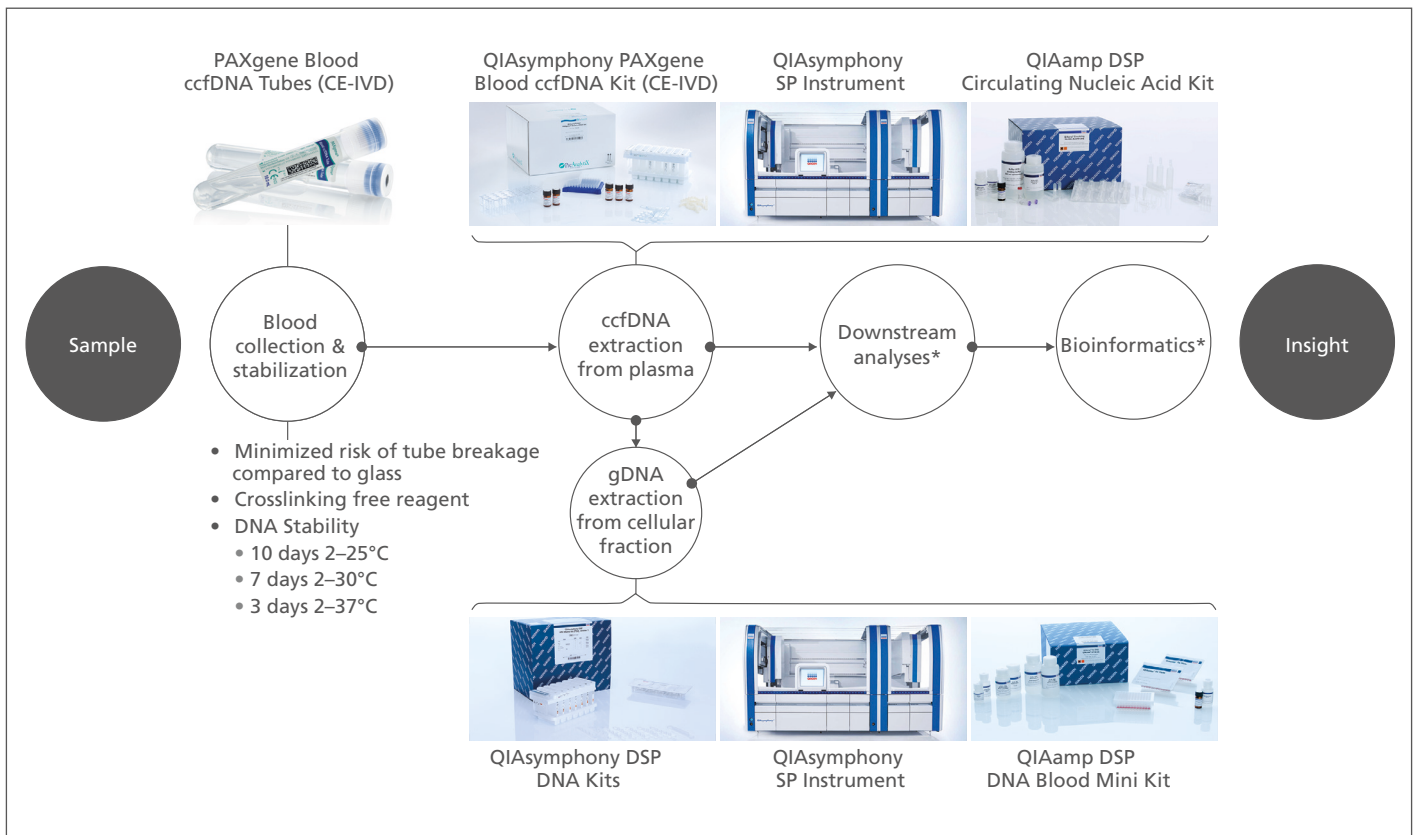


Figure 1. PAXgene Blood ccfDNA workflow (CE-IVD).

The PAXgene Blood ccfDNA Tube (CE-IVD) with the QIASymphony PAXgene Blood ccfDNA Kit (CE-IVD) and QIAGEN QIASymphony instrument (CE-IVD) have been verified and validated as an integrated workflow.

Plasma generated from whole blood collected into PAXgene Blood ccfDNA Tubes (CE-IVD) can be used to process ccfDNA automated with the QIASymphony PAXgene Blood ccfDNA Kit, or manually with the QIAamp DSP Circulating Nucleic Acid Kit. The nucleated cellular fraction or buffy coat remaining after removal of the plasma can be processed for gDNA automated with the QIASymphony DSP DNA Mini and Midi Kits, or manually with the QIAamp DSP DNA Blood Mini Kit.

*Only when used in combination with products for In Vitro Diagnostic use.

The PAXgene Blood ccfDNA Tube (CE-IVD) is CE marked for In Vitro Diagnostic (IVD) use according to the EU Regulation on in vitro diagnostic medical devices (REGULATION (EU) 2017/746) and as part of IVD development has been intensively tested in various verification and validation studies.

For short-term storage, plasma and nucleated cellular fraction can be stored at room (15–25°C) or refrigerated (2–8°C) temperatures. For long-term storage, aliquoting and freezing at –20°C or –70°C / –80°C in cryogenic tubes is recommended.

In this technical note, results from plasma and nucleated cellular fraction stability studies are shown. Stability studies with frozen plasma and nucleated cellular fraction are ongoing. Figures will be updated regularly with the latest available data. For a current listing of the performance characteristics for the PAXgene Blood ccfDNA Tube (CE-IVD), visit www.preanalytix.com.

Study Design

Blood samples were collected from consented, apparently healthy adult subjects into PAXgene Blood ccfDNA Tubes (CE-IVD) at QIAGEN (Hilden, Germany). Samples were processed and plasma separated from the nucleated cellular fraction within 4 hours after blood collection by a double centrifugation at room temperature with brake set to medium according to the tube instruction for use: 15 min $1,900 \times g$, transfer of the plasma into a secondary tube, followed by 10 min $1,900 \times g$.

For plasma stability studies one pool of plasma was prepared and aliquots stored at 25°C, 2–8°C, –20°C and –80°C. For nucleated cellular fraction stability studies, samples from individual donors were stored separately at 25°C, 2–8°C, –20°C and –80°C.

ccfDNA was extracted from the plasma with the QIASymphony PAXgene Blood ccfDNA Kit (CE-IVD) on the QIAGEN QIASymphony SP Instrument using the 2.4 ml protocol. ccfDNA yield was quantified with a validated probe-based real-time PCR assay amplifying a 66 bp fragment of the 18S rDNA gene on the QIAGEN Rotor-Gene® Q instrument using a reference gDNA standard included into each PCR run.

gDNA was extracted from nucleated cellular fraction samples with the QIASymphony DSP DNA Midi Kit on the QIASymphony SP Instrument using the 400 µl protocol. gDNA yield and purity were measured by spectrophotometry on a NanoDrop™ Spectrophotometer (Thermo Fisher Scientific), and gDNA integrity by agarose gel electrophoresis.

Results

Plasma and nucleated cellular fraction stability after storage at ambient temperatures and against repeated freezing and thawing cycles:

One plasma pool was prepared from 33 donors and aliquots were either processed immediately (T_0), stored at 25°C, stored at 2–8°C or repeatedly frozen and thawed from –20°C. Storage of up to 7 days at 25°C and up to 30 days at refrigerated, 2–8°C as well as repeated freezing and thawing from –20°C, had no or minor effect on ccfDNA yield, measured as copies 18S rDNA per ml plasma (**Figure 2A** and **B**).

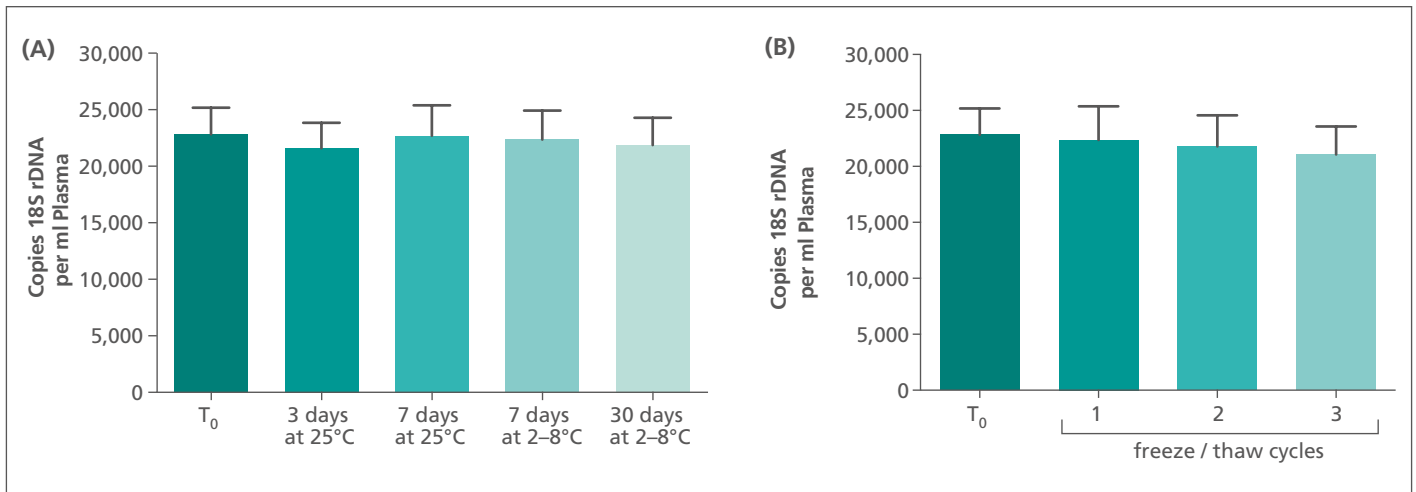


Figure 2. ccfDNA yield from plasma stored at ambient temperatures and after repeated freezing and thawing cycles.

Plasma generated from PAXgene Blood ccfDNA Tubes (CE-IVD) was processed and pooled from 33 donors within 4 hours after blood collection. ccfDNA was extracted from 20 aliquots of pooled plasma directly after processing (T_0) and after storage at 25°C, at 2–8°C or after freeze/thaw cycles as indicated using the QIASymphony PAXgene Blood ccfDNA Kit (CE-IVD) on the QIASymphony SP Instrument (2.4 ml protocol) and analysed for the 18S rDNA target gene. Mean values with standard deviations of total number of copies 18S rDNA per ml plasma (including stabilization reagent) are shown, $n = 20$.

(A) Storage at 25°C or 2–8°C for the indicated time.

(B) One, two or three freeze and thaw cycles from –20°C.

After removal of the plasma, the nucleated cellular fraction from the 33 individual donor samples was tested for genomic DNA stability when stored at 25°C and 2–8°C, and against repeated cycles of freezing and thawing from –20°C.

Storage of 3 days at 25°C, up to 7 days at refrigerated 2–8°C, or up to three times repeated freezing and thawing from –20°C had no effect on genomic DNA yield, purity, or integrity (**Figure 3** and **4**).

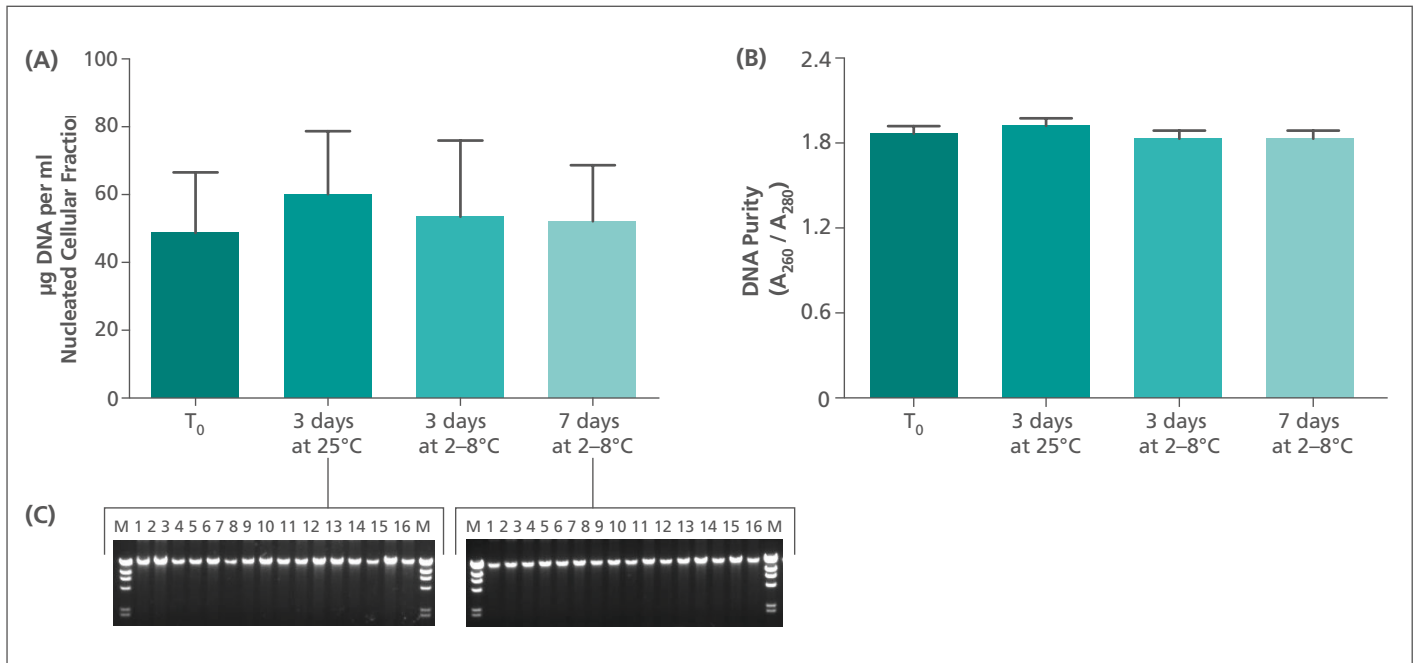


Figure 3. Yield, purity and integrity of genomic DNA purified from nucleated cellular fraction stored at ambient temperatures.

Blood collected into PAXgene Blood ccfDNA Tubes (CE-IVD) from 33 donors was processed directly (<4 hours) after blood collection. Genomic DNA was extracted from the nucleated cellular fraction of individual samples directly after processing (T₀) or after storage for 3 days at 25°C and up to 7 days at 2–8°C with the QIAasymphony DSP DNA Midi Kit on the QIAasymphony SP Instrument using the 400 µl protocol. Yield and purity were measured by spectrophotometry on a NanoDrop spectrophotometer, DNA integrity by agarose gel electrophoresis.

(A) gDNA yield in µg DNA per ml nucleated cellular fraction. Values are means with standard deviation, n = 33.

(B) gDNA purity (A₂₆₀ / A₂₈₀). Values are means with standard deviation, n = 33.

(C) Examples for DNA integrity shown for 16 donors (1–16); nucleated cellular fraction was stored for 3 days at 25°C and 7 days at 2–8°C; a Lambda x Hind III marker was loaded into lane “M”. The upper band of this marker represents a DNA fragment of 23 kb.

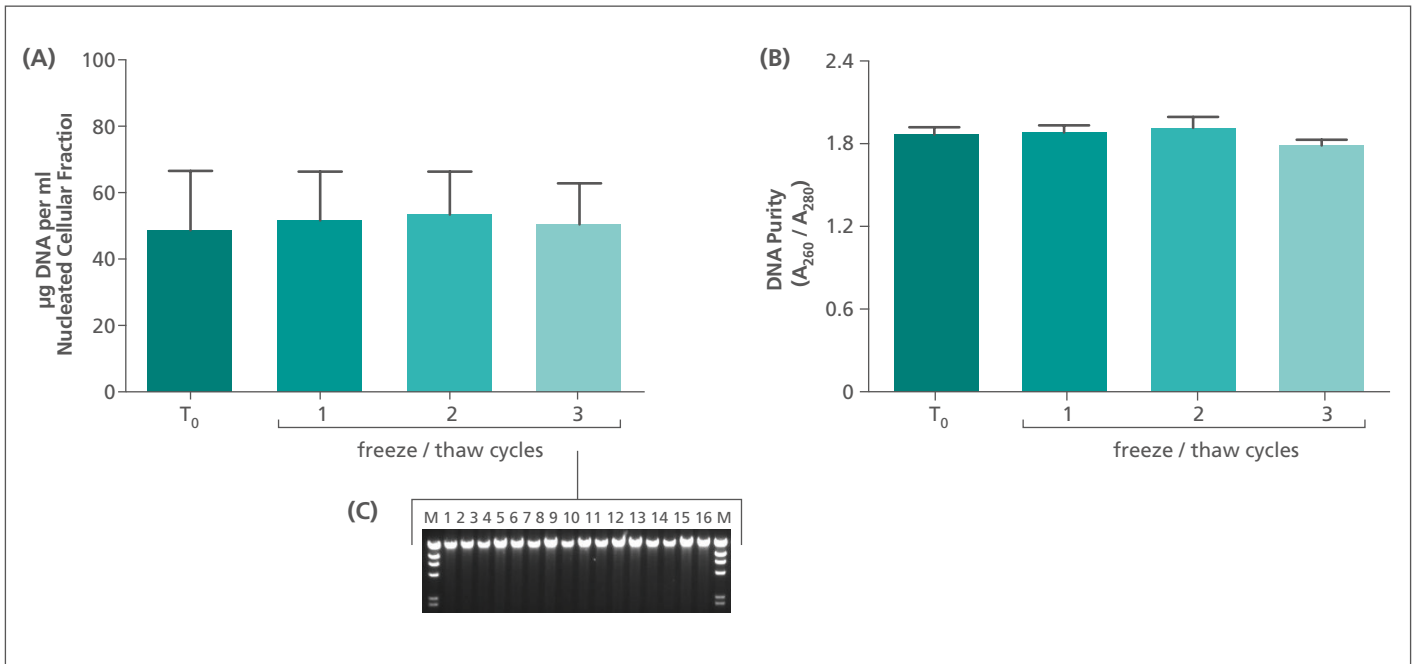


Figure 4. Yield, purity and integrity of genomic DNA purified from nucleated cellular fraction after repeated freezing and thawing cycles.

Blood collected into PAXgene Blood ccfDNA Tubes (CE-IVD) from 33 donors was processed directly (<4 hours) after blood collection. Genomic DNA was extracted from the nucleated cellular fraction of individual samples directly after processing (T₀) or after one, two or three freezing and thawing from -20°C cycles. DNA was purified with the QIASymphony DSP DNA Midi Kit (CE-IVD) on the QIASymphony SP Instrument using the 400 µl protocol. Yield and purity were measured by spectrophotometry on a NanoDrop spectrophotometer, DNA integrity by agarose gel electrophoresis.

(A) gDNA yield in µg DNA per ml nucleated cellular fraction. Values are means with standard deviation, n = 33.

(B) gDNA purity (A₂₆₀ / A₂₈₀). Values are means with standard deviation, n = 33.

(C) Examples for DNA integrity shown for 16 Donors (1–16); nucleated cellular fraction was three times frozen and thawed; a Lambda x Hind III marker was loaded into lane "M". The upper band of this marker represents a DNA fragment of 23 kb.

Frozen plasma and nucleated cellular fraction long-term stability:

To determine the stability of ccfDNA in plasma stored for long-term frozen at -20°C or -80°C , one large plasma pool was generated with blood samples from 42 donors drawn into PAXgene Blood ccfDNA Tubes (CE-IVD) within 4 hours after blood collection. Aliquots of the plasma pool are stored at -20°C and -80°C and will be tested at least once per year for total ccfDNA yield by PreAnalytiX. This long-term storage study was started in 2019 and is designed to last for up to 20 years.

Intermediate storage timepoint one year storage at -20°C or -80°C did not show reduced ccfDNA yield measured as copies 18S rDNA per ml plasma (**Figure 5**).

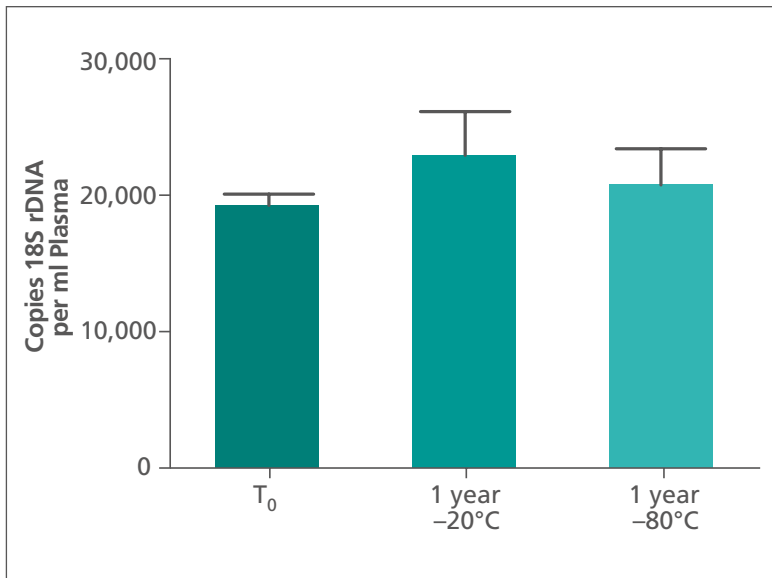


Figure 5. ccfDNA yield from plasma stored long-term at -20°C and -80°C .

Plasma generated from PAXgene Blood ccfDNA Tubes (CE-IVD) were processed and pooled from 42 donors within 4 hours after blood collection. Aliquots of the plasma pool were stored frozen at -20°C and -80°C . ccfDNA was extracted from 20 aliquots directly after processing (T_0) and after storage as indicated using the QIASymphony PAXgene Blood ccfDNA Kit (CE-IVD) on the QIASymphony SP Instrument (2.4 ml protocol) and analysed for the 18S rDNA target gene. Mean values with standard deviations of total number of copies 18S rDNA per ml plasma (including stabilization reagent) are shown, $n = 20$.

To determine the stability of gDNA after removal of the plasma, the nucleated cellular fraction from the 42 individual donors was stored for long-term frozen at -20°C or -80°C . The impact of storage time on gDNA yield, purity, and integrity is planned to be tested at least once a year.

The first intermediate storage timepoints showed a moderate decrease in yield after 6 months storage compared to the initial test-timepoint for samples stored at -20°C and at -80°C . After this initial decrease, yield remained constant at the next test-timepoint of 12 months (**Figure 6 A**). Storage time had no effect on genomic DNA purity and integrity (**Figure 6 B and C**).

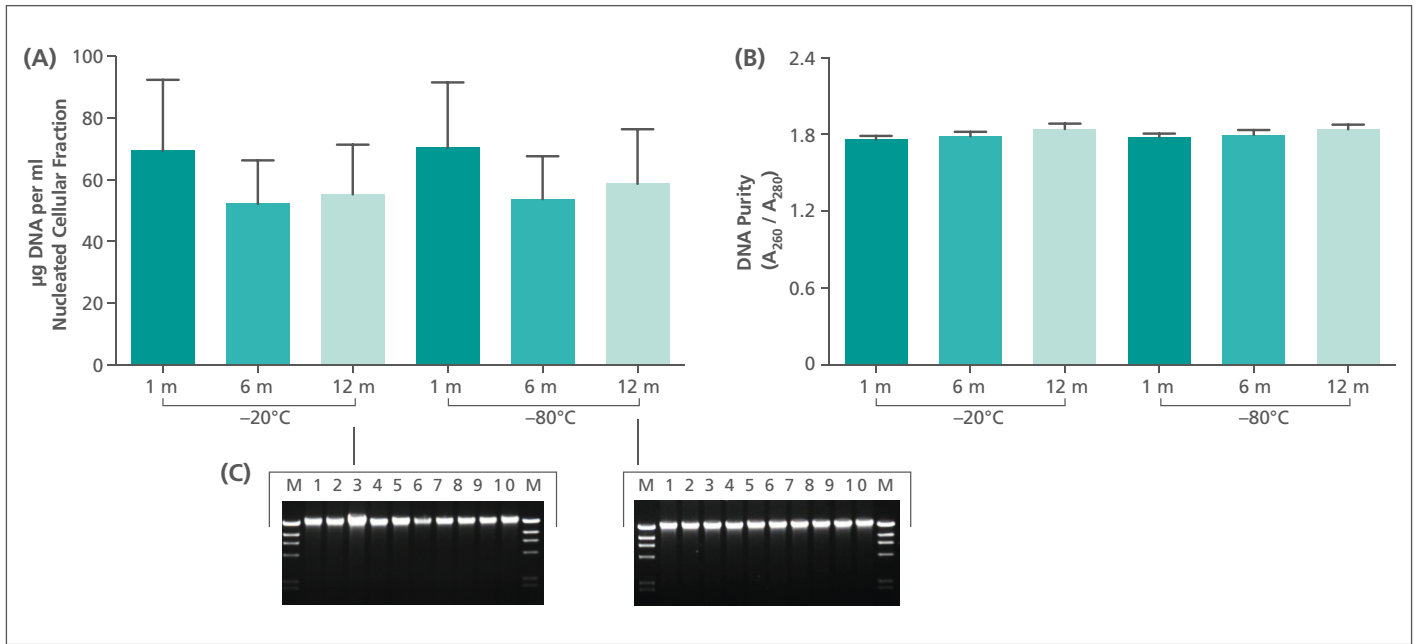


Figure 6. Yield, purity and integrity of genomic DNA purified from nucleated cellular fraction stored long-term at -20°C and -80°C .

Blood collected into PAXgene Blood ccfDNA Tubes (CE-IVD) from 42 donors was processed within 4 hours after blood collection. Aliquots of nucleated cellular fraction from each donor were stored frozen at -20°C and -80°C . Genomic DNA was extracted after storage with the QIASymphony DSP DNA Midi Kit (CE-IVD) on the QIASymphony SP Instrument using the 400 μl protocol. Yield and purity were measured by spectrophotometry on a NanoDrop spectrophotometer, DNA integrity by agarose gel electrophoresis.

(A) gDNA yield in μg DNA per ml nucleated cellular fraction. Values are means with standard deviation, $n = 42$.

(B) gDNA purity (A_{260} / A_{280}). Values are means with standard deviation, $n = 42$.

(C) Examples for DNA integrity shown for 10 Donors (1–10); nucleated cellular fraction was stored for 12 months at -20°C and at -80°C ; a Lambda x Hind III marker was loaded into lane "M". The upper band of this marker represents a DNA fragment of 23 kb.

Conclusion

The objective of this study was to analyse the stability of ccfDNA in plasma and gDNA in the nucleated cellular fraction isolated from PAXgene Blood ccfDNA Tubes (CE-IVD) under certain storage conditions including storage at room and refrigerated temperatures, long-term storage when frozen to -20°C or -80°C , and repeated freezing and thawing cycles.

Study results demonstrated that ccfDNA and gDNA remains stable in plasma and nucleated cellular fraction, respectively, when stored for up to 3 days at 25°C , up to 7 days at $2-8^{\circ}\text{C}$ and after up to 3 freeze and thaw cycles. For a current listing of the performance characteristics for the PAXgene Blood ccfDNA Tube (CE-IVD), visit www.preanalytix.com. Although data indicate stability of ccfDNA in plasma at ambient temperatures, it is recommended to use plasma for ccfDNA purification as soon as possible after processing. Longer storage time should be avoided, but plasma should be frozen at -20°C or -80°C when a delay until further processing is anticipated.

Long-term storage studies are still ongoing at PreAnalytiX. Results indicate stability of ccfDNA in plasma stored at -20°C and -80°C for at least 12 months. In the nucleated cellular fraction, when stored at -20°C and -80°C , genomic DNA is stable up to 12 months. For a current listing of the performance characteristics for the PAXgene Blood ccfDNA Tube (CE-IVD), visit www.preanalytix.com.

For optimal results it is highly recommended to follow the instructions for use for the PAXgene Blood ccfDNA Tube (CE-IVD).

Products used

Product	Catalog No.
PAXgene Blood ccfDNA Tube (CE-IVD) (100)	768165
QIASymphony PAXgene Blood ccfDNA Kit (CE-IVD) (192)	768566
QIASymphony DSP DNA Midi Kit (96) (QIAGEN)	937255
QIASymphony SP instrument (QIAGEN)	9001297
QIAamp DSP Circulating Nucleic Acid Kit (50) (QIAGEN)	61504
Rotor-Gene Q instrument (QIAGEN)	9001550



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Technical Note

PAXgene® Blood ccfDNA Tube (CE-IVD) Tube Robustness Studies

The objective of these studies was to test tube robustness for the PAXgene Blood ccfDNA Tube (CE-IVD). In the studies, the preanalytical steps including handling, storage, processing and documentation were conducted according to ISO 20186-2:2019 and ISO 20186-3:2019 (Molecular in vitro diagnostic examinations – Specifications for pre-examination processes for venous whole blood – Part 2: Isolated genomic DNA and Part 3: Isolated circulating cell-free DNA from plasma).

Introduction

The PAXgene Blood ccfDNA Tube (CE-IVD) (PAXgene Tube) is a sterile, single use, plastic, evacuated blood collection tube with a circulating, cell-free DNA (ccfDNA) stabilization additive (1.5 ml of liquid additive) with a nominal blood draw volume of 10 ml. The tube additive is non-crosslinking, free of formaldehyde-releasing substances, stabilizes blood cells and prevents apoptosis. Blood filled in PAXgene Blood ccfDNA Tubes can be stored for up to 10 days at 2–25°C, up to 7 days at 2–30°C, or up to 3 days at 2–37°C prior to processing. Plasma is separated from the cellular fraction by a double centrifugation, first for 15 minutes at 1,600–3,000 × *g*, then transfer of the plasma into a secondary tube, followed by centrifugation of the plasma for another 10 minutes at 1,600–3,000 × *g*. As an alternative after a first centrifugation for 15 minutes at 3,000 × *g* the tube can be directly placed on the QIAGEN® QIAasymphony® SP instrument for automated ccfDNA extraction.

Plasma generated from whole blood collected into PAXgene Blood ccfDNA Tubes (CE-IVD) can be used to process ccfDNA automated with the PreAnalytiX® QIAasymphony PAXgene Blood ccfDNA Kit (CE-IVD), or manually with the QIAGEN QIAamp® DSP Circulating Nucleic Acid Kit. The cellular fraction after removal of the plasma can be processed for genomic DNA (gDNA) automated with the QIAGEN QIAasymphony DSP DNA Mini and Midi Kits or manually with the QIAGEN QIAamp DSP DNA Blood Mini Kit.

PreAnalytiX developed the PAXgene Blood ccfDNA blood collection tube and purification kits to standardize the preanalytical workflow in order to minimize post-collection changes in circulating, cell-free DNA (ccfDNA) and gDNA profiles of whole blood caused by preanalytical variables (**Figure 1**).

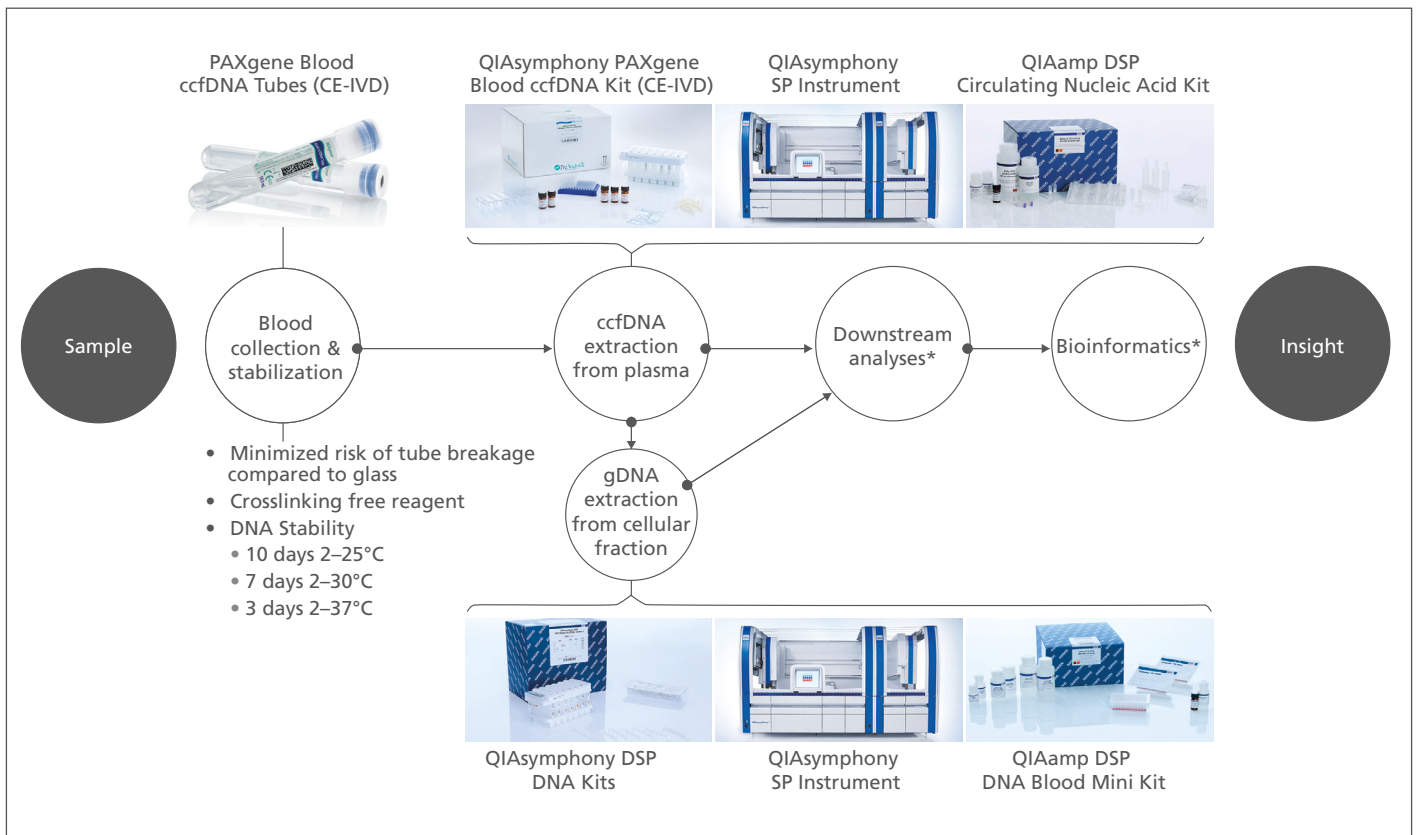


Figure 1. PAXgene Blood ccfDNA workflow (CE-IVD).

The PAXgene Blood ccfDNA Tube (CE-IVD) with the QIASymphony PAXgene Blood ccfDNA Kit (CE-IVD) and QIAGEN QIASymphony instrument (CE-IVD) have been verified and validated as an integrated workflow.

Plasma generated from whole blood collected into PAXgene Blood ccfDNA Tubes (CE-IVD) can be used to process ccfDNA automated with the QIASymphony PAXgene Blood ccfDNA Kit, or manually with the QIAamp DSP Circulating Nucleic Acid Kit. The nucleated cellular fraction or buffy coat remaining after removal of the plasma can be processed for gDNA automated with the QIASymphony DSP DNA Mini and Midi Kits, or manually with the QIAamp DSP DNA Blood Mini Kit.

*Only when used in combination with products for In Vitro Diagnostic use.

The PAXgene Blood ccfDNA Tube (CE-IVD) is CE marked for In Vitro Diagnostic (IVD) use according to the EU Regulation on in vitro diagnostic medical devices (REGULATION (EU) 2017/746) and as part of IVD development has been intensively tested in various verification and validation studies.

According to the PAXgene Blood ccfDNA Tube instructions for use the tube needs to be filled to its maximum stated draw volume by continuing blood collection until the vacuum is exhausted and blood has stopped flowing into the tube before removing the tube from the holder. After blood collection, the PAXgene Blood ccfDNA Tube (CE-IVD) needs to be inverted gently 8 times to mix the blood with the additive.

However, users might invert the tube fewer, more times or with a delay. A phlebotomy can be interrupted prematurely before the vacuum is exhausted and blood samples might contain elevated level of potentially interfering endogenous substances. In this technical note we present the results from robustness studies to determine how inappropriate handling, and elevated level of endogenous substances influence the DNA stabilization and purification characteristics of whole blood samples collected into PAXgene Blood ccfDNA Tubes (CE-IVD).

Study Design

Blood samples were collected from consented, apparently healthy adult subjects into PAXgene Blood ccfDNA Tubes (CE-IVD) at QIAGEN (Hilden, Germany). For each condition tested, one sample was processed immediately within 4 hours after blood collection and the second sample was stored for 7 days at 30°C, the longest storage duration specified for an elevated temperature above room temperature.

Plasma was generated according to the tube handbook by a double centrifugation at room temperature with brake set to medium: 15 min 1,900 × *g*, transfer of the plasma into a secondary tube, followed by 10 min 1,900 × *g*.

ccfDNA was extracted from the plasma with the QIASymphony PAXgene Blood ccfDNA Kit (CE-IVD) on the QIAGEN QIASymphony SP Instrument using the 2.4 or 4.8 ml protocol. ccfDNA yield was quantified with a validated probe-based real-time PCR assay amplifying a 66 bp fragment of the 18S rDNA gene on the QIAGEN Rotor-Gene® Q instrument using a reference gDNA standard included into each PCR run. Because the amount of ccfDNA can vary up to 40 fold between individual donors, total copy numbers for ccfDNA are shown as medians instead of mean values.

After removal of the plasma gDNA was extracted from the nucleated cellular fraction with the QIASymphony DSP DNA Midi Kit on the QIASymphony SP Instrument using the 400 µl protocol. gDNA yield and purity were measured by spectrophotometry on a NanoDrop™ Spectrophotometer (Thermo Fisher Scientific), and gDNA integrity by agarose gel electrophoresis.

Conditions tested

Study 1: Tube Underfilling

Six tubes were drawn from each donor. For reference, two tubes were drawn until the vacuum was exhausted (100%). For two tubes the blood flow was interrupted when the tubes were $\frac{3}{4}$ full (25% underfilling) and for two tubes when they were half full (50% underfilling). One tube from each filling condition was processed within 4 hours after blood collection. The second tube was stored for 7 days at 30°C.

Study 2: Tube Inappropriate Mixing

Eight tubes were drawn from each donor. As reference, two tubes were inverted 8 times directly after draw according to the handbook. An additional two tubes per donor were inverted 2 times or 20 times directly after draw, or 8 times but with a 5 minute delay. One tube from each mixing condition was processed within 4 hours after blood collection. The second tube was stored for 7 days at 30°C.

Study 3: Endogenous Interfering Substances

Up to ten tubes were drawn from each donor. Directly after blood draw, the tubes were spiked and thoroughly mixed with selected substances from **Table 1** based on recommendations for physiological and spike concentrations in CLSI guideline EP07 (Interference Testing in Clinical Chemistry, 3rd Edition). Spike in of plasma free (pf) Hemoglobin was conducted according to an internally validated method. Depending on solubility each substance was dissolved in water or ethanol and then spiked in, or spiked in directly as a powder or emulsion. As controls to evaluate the solvents, a tube from each donor was spiked with water and one with ethanol (**Table 1**).

From each tube half of the sample was removed and processed immediately, within 4 hours after blood collection. The other half was stored for 7 days at 30°C until processing.

Substance	Physiological concentration in blood [mg/ml*]	Stock concentration	Spike-In mass or volume / tube [mg]	Final concentration [mg/ml]
Water (control)/H ₂ O	–	–	100 (µl)	–
Ethanol (control)/EtOH	–	–	100 (µl)	–
Albumin	35–52*	Powder	170	<52
Bilirubin, unconjugated	0.002–0.008*	6.8 mg/ml in EtOH	1.5	<0.152
Cholesterol, total	<2*	100 mg/ml in EtOH	22	<4.2
Glucose	0.74–1.00*	150 mg/ml in H ₂ O	15	<0.89
pf Hemoglobin	0.1–0.3	50 mg/ml in H ₂ O	5	<0.6
Triglycerides, total	<1.5*	20% fat emulsion	25	<4

Table 1. Endogenous interfering substances spiked into whole blood

* CLSI guideline (EP07 -Interference Testing in Clinical Chemistry, 3rd Edition)

Results

Study 1: Tube Underfilling

PAXgene Blood ccfDNA Tubes (CE-IVD) were underfilled by premature interruption of blood flow. The tube has a nominal fill volume of 10 ml. Therefore, underfilling by 25 and 50% (75 and 50% filling) led to a draw volume of 7.5 and 5 ml whole blood respectively.

Since the tube includes 1.5 ml stabilization reagent, lower fill-volume leads to an increased dilution of the plasma. When the dilution effect was arithmetically deducted, total yield in copy numbers 18S rDNA molecules per ml human plasma were only slightly reduced in underfilled tubes (**Figure 2A**). Moreover, the ability of the liquid additive to prevent DNA release over a storage time of 7 days at 30°C was not affected (**Figure 2B**).

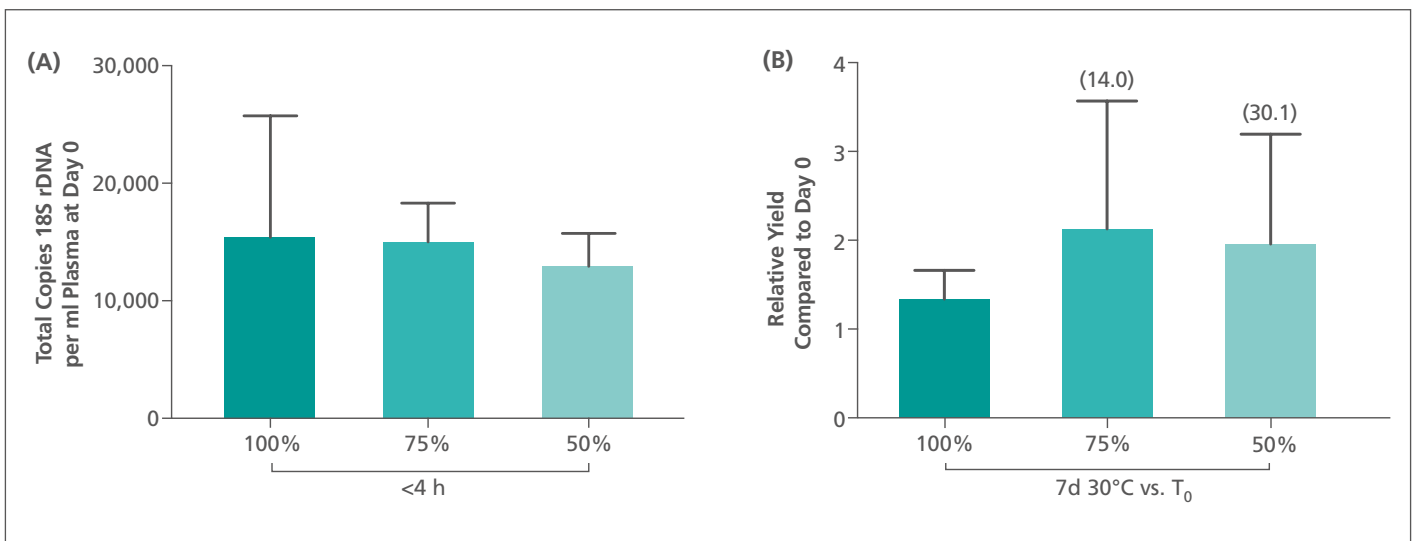


Figure 2. Impact of incorrect filling on ccfDNA yield and prevention of DNA release.

Correctly filled and underfilled (filled to 75 and 50%) PAXgene Blood ccfDNA Tubes (CE-IVD) were processed from 14 donors directly after blood collection (<4 h) and after 7 days storage at 30°C. ccfDNA from plasma was extracted with the QIASymphony PAXgene Blood ccfDNA Kit (CE-IVD) on the QIASymphony SP Instrument (2.4 ml protocol) and analyzed for the 18S rDNA target gene.

- (A)** ccfDNA yield: Total number of copies 18S rDNA per ml pure plasma (dilution effect from PAXgene Tube additive deducted). Values are medians with 95% confidence, n = 16.
- (B)** Prevention of DNA release: relative yield after storage for 7 days at 30°C compared to processing within 4 hours. Values are means with standard deviation, n = 16 (100%) and 15 (one outlier according to Graf-Henning-outlier test excluded for 75% and 50%; value is shown in brackets).

Genomic DNA isolated from the nucleated cellular fraction was of high yield, purity, and integrity for all donors and all filling conditions tested (**Figure 3**).

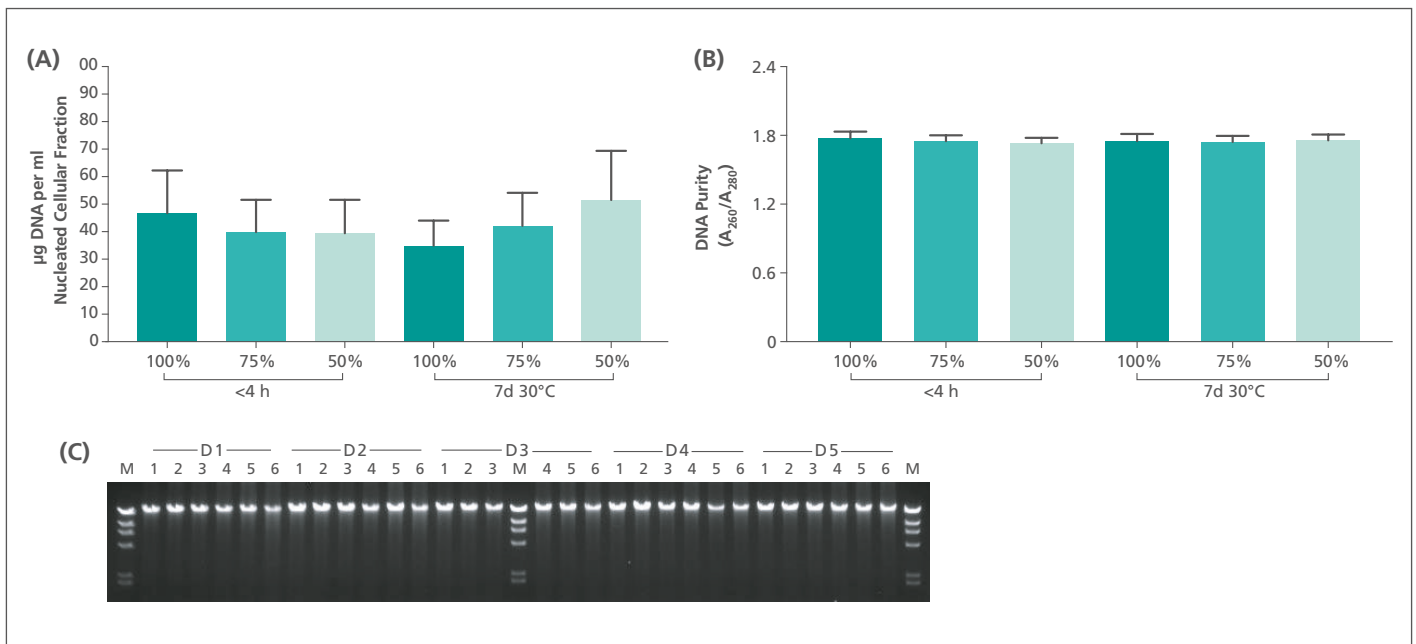


Figure 3. Impact of incorrect filling on gDNA yield, purity and integrity.

Yield, purity, and integrity of genomic DNA from the nucleated cellular fraction of correctly filled and underfilled (filled to 75 or 50%) PAXgene Blood ccfDNA Tubes, processed from 16 donors directly after blood collection (<4 h) and after 7 days storage at 30°C. DNA from the nucleated cellular fraction was extracted with the QIASymphony DSP DNA Midi Kit on the QIASymphony SP Instrument using the 400 μ l protocol. Yield and purity were measured by spectrophotometry on a NanoDrop spectrophotometer, DNA integrity by agarose gel electrophoresis.

- (A)** gDNA yield in μ g DNA per ml nucleated cellular fraction. Values are means with standard deviation, $n = 16$.
(B) gDNA purity (A_{260} / A_{280}). Values are means with standard deviation, $n = 16$.
(C) Examples for DNA integrity shown for five Donors (D1–D5). 1–3: DNA from 100, 75 and 50% filled tube at timepoint <4 hours after collection; 4–6: DNA from 100, 75 and 50% filled tube at timepoint 7 days at 30°C; a Lambda x Hind III marker was loaded into lane “M”. The upper band of this marker represents a DNA fragment of 23 kb.

Study 2: Tube Inappropriate Mixing

In order to efficiently mix stabilization reagent and blood, the PAXgene Blood ccfDNA Tube (CE-IVD) handbook states to gently invert the tube 8 times after the tube is completely filled. To test the impact of inappropriate tube handling altogether eight tubes were drawn from each donor. Two tubes – one for immediate processing, one for storage – were drawn for each condition tested. Conditions tested were mixing 2 and 20 times, and 8 times but with a 5 minute delay after blood collection. A control set of tubes was gently inverted 8 times immediately after blood collection.

The investigated inappropriate mixing conditions had no effects or minor effects on total ccfDNA yield when plasma was processed within 4 hours after blood collection (**Figure 4A**). DNA release was prevented in samples stored for 7 days at 30°C for all conditions tested (**Figure 4B**).

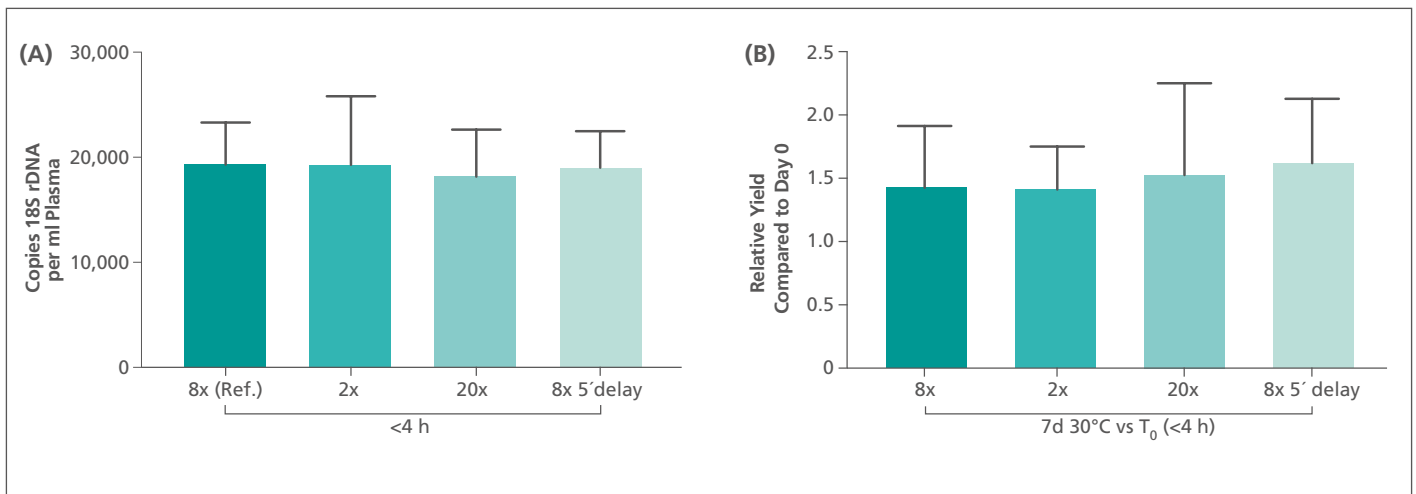


Figure 4. Impact of inappropriate mixing on ccfDNA yield and prevention of DNA release.

Correctly mixed (8x inversion) and inappropriately mixed (2x, 20x or 8x inverted with 5 minute delay) PAXgene Blood ccfDNA Tubes (CE-IVD) were processed from 13 donors directly after blood collection (<4 h) and after 7 days storage at 30°C. ccfDNA from plasma was extracted with the QIASymphony PAXgene Blood ccfDNA Kit (CE-IVD) on the QIASymphony SP Instrument (2.4 ml protocol) and analyzed for the 18S rDNA target gene.

- (A)** ccfDNA yield: Total number of copies 18S rDNA per ml plasma from a PAXgene Tube.
Values are medians with 95% confidence, n = 13.
- (B)** Prevention of DNA release: relative yield after storage for 7 days at 30°C compared to processing within 4 hours.
Values are means with standard deviation, n = 13.

Genomic DNA isolated from the nucleated cellular fraction was of high yield, purity, and integrity for all donors and all mixing conditions tested (**Figure 5**).

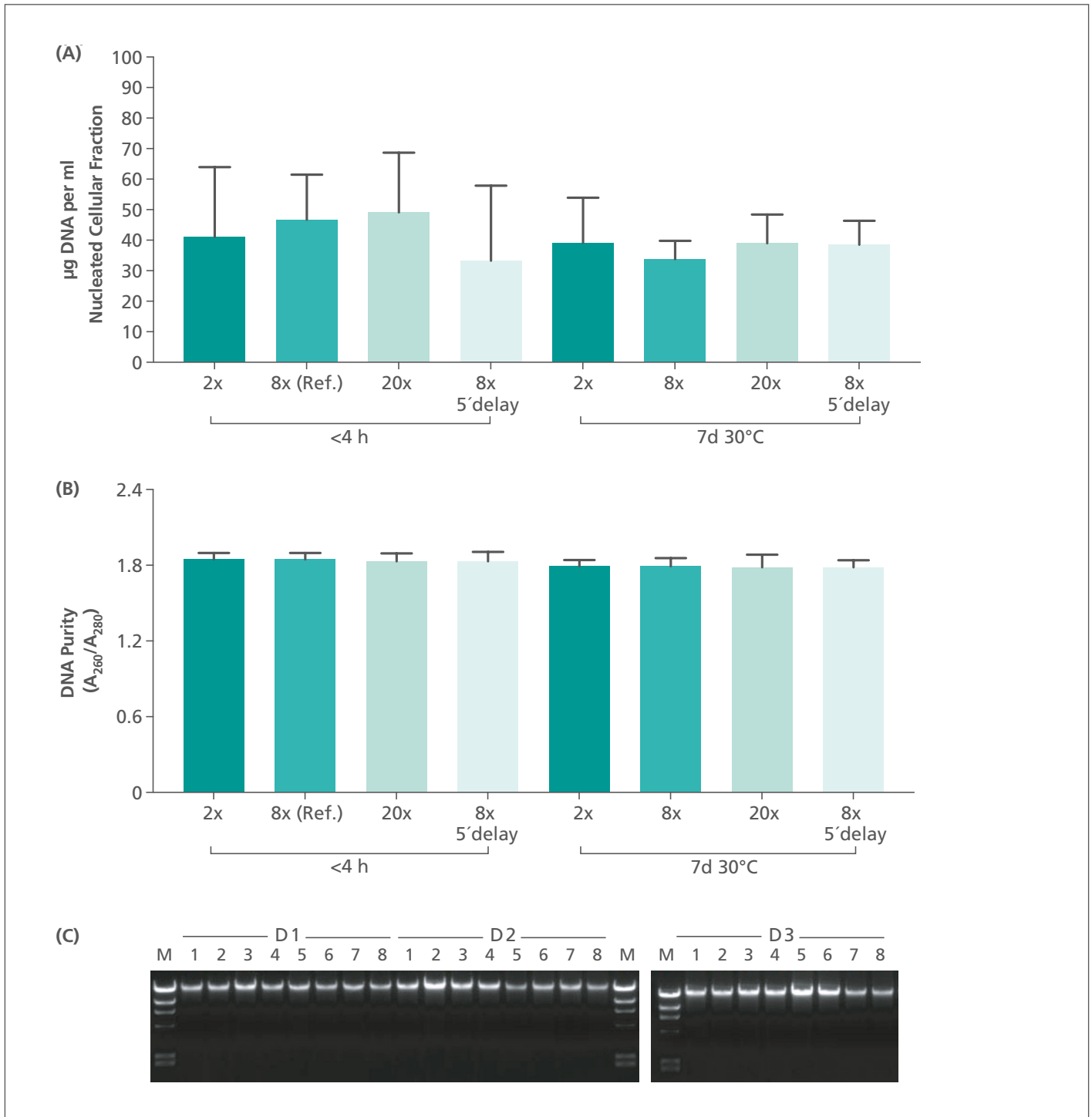


Figure 5. Impact of inappropriate mixing on gDNA yield, purity and integrity.

Correctly mixed (8x inversion) and inappropriately mixed (2x, 20x or 8x inverted with 5 minute delay) PAXgene Blood ccfDNA Tubes (CE-IVD) were processed from 13 donors directly after blood collection (<4 h) and after 7 days storage at 30°C. DNA from the nucleated cellular fraction was extracted with the QIASymphony DSP DNA Midi Kit on the QIASymphony SP Instrument using the 400 µl protocol. Yield and purity were measured by spectrophotometry on a NanoDrop spectrophotometer, DNA integrity by agarose gel electrophoresis.

(A) gDNA yield in µg DNA per ml nucleated cellular fraction. Values are means with standard deviation, n = 13.

(B) gDNA purity (A_{260}/A_{280}). Values are means with standard deviation, n = 13.

(C) Examples for DNA integrity shown for three Donors (D1–D3). 1–4: DNA from tubes mixed 8x (reference), 2x, 20x, 8x with 5 min delay at timepoint <4 hours after collection; 5–8: DNA from 8x (reference), 2x, 20x, 8x with 5 min delay at timepoint 7 days at 30°C; a Lambda x Hind III marker was loaded into lane “M”. The upper band of this marker represents a DNA fragment of 23 kb.

Study 3: Endogenous Interfering Substances

To verify that elevated level of endogenous substances in blood do not interfere with DNA extraction, quality, and functional performance, blood from 10 healthy, consented donors was collected into PAXgene Blood ccfDNA Tubes (CE-IVD) and potentially interfering endogenous substances were spiked into the blood within 2 hours.

Substances chosen for testing are representative of commonly occurring potentially interfering constituents of blood. The substances did not have an impact on ccfDNA yield when samples were processed directly after spike-in within 4 hours after blood collection (**Figure 6A**). DNA release during blood storage was prevented, because the relative ccfDNA yield after storage for 7 days at 30°C, compared to corresponding day 0 samples was not or only moderately increased compared to the no spike (water and ethanol) controls (**Figure 6B**).

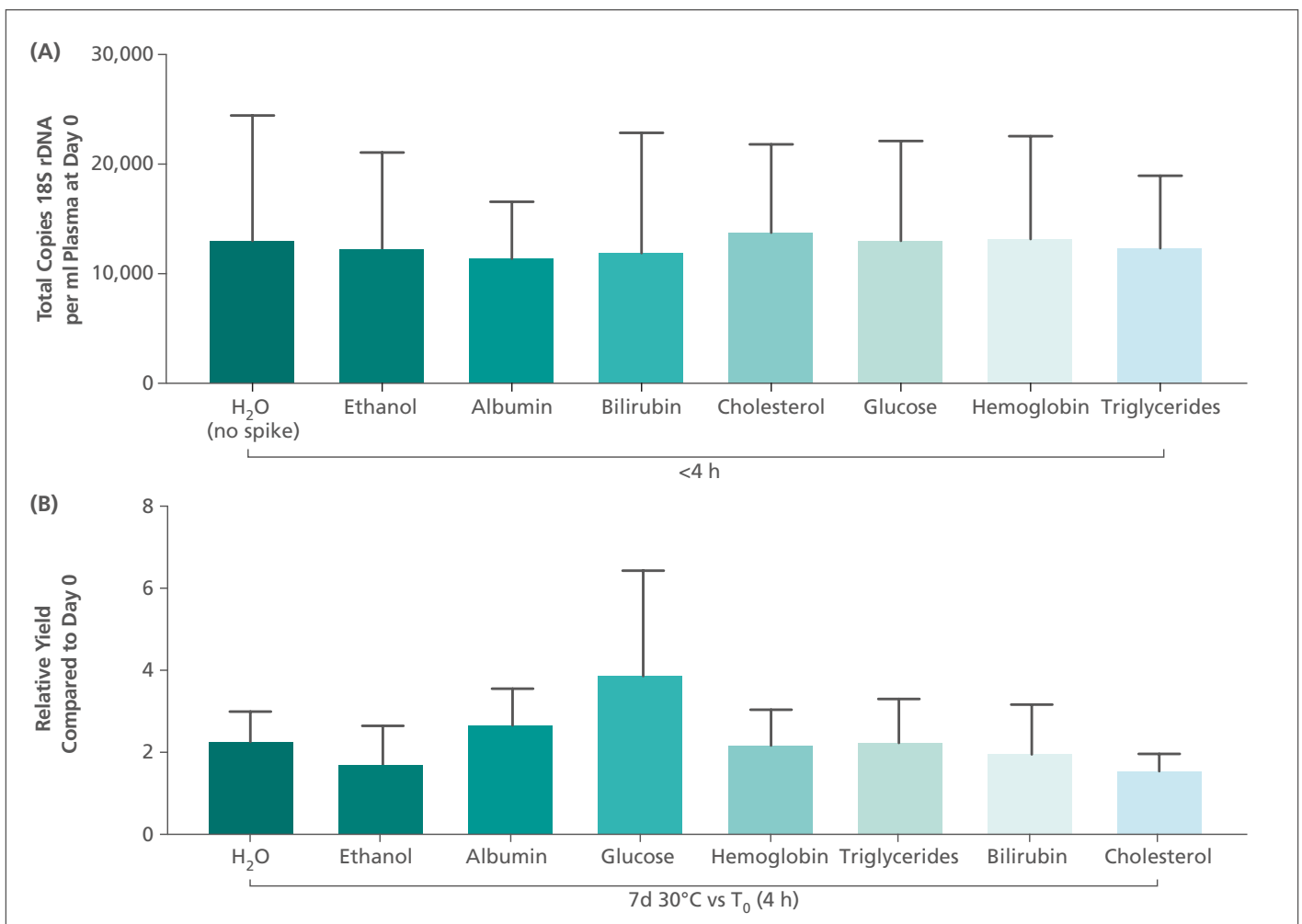


Figure 6. Impact of endogenous interference substances on ccfDNA yield.

Filled PAXgene Blood ccfDNA Tubes (CE-IVD) were spiked with interfering substances to reach target concentration in mg per ml of blood according to CLSI guideline EP07 (**Table 1**) and processed directly (<4 hours) and after 7 days storage at 30°C. ccfDNA from plasma was extracted with the QIASymphony PAXgene Blood ccfDNA Kit (CE-IVD) on the QIASymphony SP Instrument (2.4 ml protocol) and analyzed for the 18S rDNA target gene.

- (A)** ccfDNA yield: Total number of copies 18S rDNA per ml plasma from a PAXgene Tube. Values are medians with 95% confidence, n = 10.
- (B)** Prevention of DNA release: relative yield after storage for 7 days at 30°C compared to processing within 4 hours. Values are means with standard deviation, n = 10.

Genomic DNA isolated from the nucleated cellular fraction was not affected with regard to yield, purity, and integrity by the interfering substances tested (**Figure 7**).

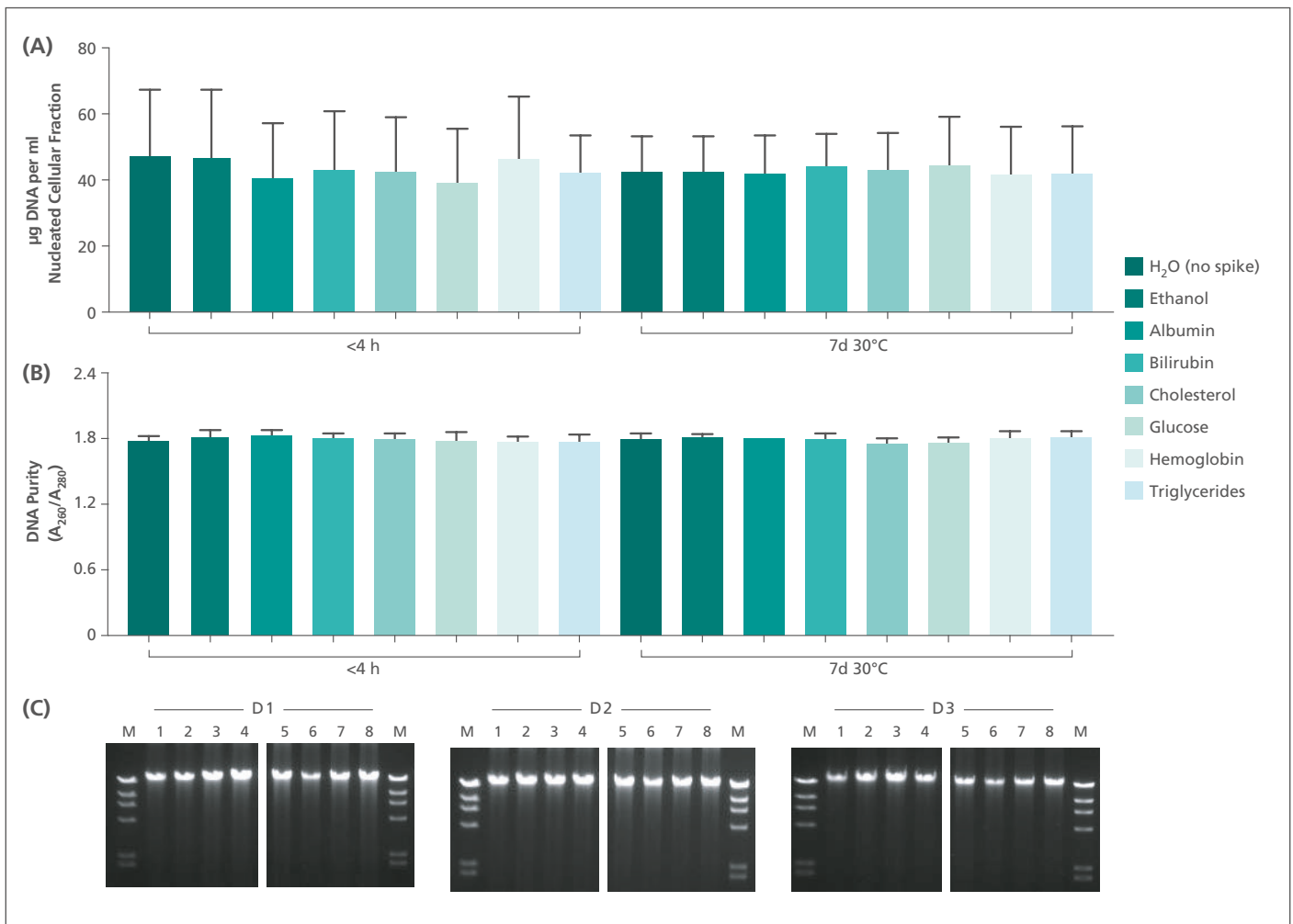


Figure 7. Impact of endogenous interference substances on gDNA yield, purity and integrity.

Filled PAXgene Blood ccfDNA Tubes (CE-IVD) were spiked with interfering substances to reach target concentration in mg per ml of blood according to CLSI guideline EP07 and processed directly (<4 hours) and after 7 days storage at 30°C. DNA was extracted from the nucleated cellular fraction with the QIAasymphony DSP DNA Midi Kit on the QIAasymphony SP Instrument using the 400 µl protocol. Yield and purity were measured by spectrophotometry on a NanoDrop spectrophotometer, DNA integrity by agarose gel electrophoresis.

(A) gDNA yield in µg DNA per ml nucleated cellular fraction. Values are means with standard deviation, n = 10.

(B) gDNA purity (A₂₆₀/A₂₈₀). Values are means with standard deviation, n = 10.

(C) Examples for DNA integrity shown for three Donors (D1–D3); 1: Cholesterol, 2: Glucose, 3: Hemoglobin, 4: Triglycerides, 5: Water (no spike in control), 6: Ethanol (control – solvent for bilirubin and cholesterol), 7: Albumin, 8: Bilirubin. Lambda x Hind III marker was loaded into lane “M”. The upper band of this marker represents a DNA fragment of 23 kb.

Conclusion

The objective of these studies was to analyze the impact of inappropriate handling, such as underfilling, incomplete, excessive or delayed mixing, as well as elevated level of endogenous substances in blood on the ability of the PAXgene Blood ccfDNA Tube (CE-IVD) reagent to prevent release of DNA from blood cells and purification of ccfDNA from plasma or gDNA from the nucleated cellular fraction.

Taken together, the study results demonstrated the robustness of the PAXgene Blood ccfDNA (CE-IVD) workflow. Inappropriate handling, such as underfilling, incomplete, excessive or delayed mixing, as well as elevated level of the physiological substances tested in these studies did not impair purification of ccfDNA from plasma and gDNA from the nucleated cellular fraction. Moreover, the liquid additive efficiently prevented release of DNA by blood cells in the same range of experimental conditions.

For optimal results it is highly recommended to follow the instructions for use in the PAXgene Blood ccfDNA Tube (CE-IVD) handbook. Users must validate the use of the device for their specific molecular diagnostic assay.

Products used

Product	Catalog No.
PAXgene Blood ccfDNA Tube (CE-IVD) (100)	768165
QIASymphony PAXgene Blood ccfDNA Kit (CE-IVD) (192)	768566
QIASymphony DSP DNA Midi Kit (96) (QIAGEN)	937255
QIASymphony SP instrument (QIAGEN)	9001297
QIAamp DSP Circulating Nucleic Acid Kit (50) (QIAGEN)	61504
Rotor-Gene Q instrument (QIAGEN)	9001550



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